IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION

I, Paul David Churchill Clarke, BA. MITI, translator to Taylor & Meyer of 20 Kingsmead Road, London SW2 3JD, England, do solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and German languages;
2. That the following is a true translation made by me into the English language of German Priority Text Application No. 100 11 922.0;
3. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed, this 28th day of December 2002

P.D.C Clarke

Harrogate, HG2 0HA, England

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION OF

MÖCKEL et al.

Group Art Unit: 1652

Appln. No.: 09/715,035

Examiner: C. FRONDA

Filed: November 20, 2000

Title: NUCLEOTIDE SEQUENCES ENCODING FOR THE *pfka* GENE

January 28, 2003

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TRANSMITTAL OF CERTIFIED TRANSLATIONS OF PRIORITY DOCUMENTS

Hon. Commissioner of Patents
Washington, D.C. 20231

Sir:

Further to the applicants' response of December 9, 2002, and the official action of September 9, 2002 (see page 2, Paragraph No. 5), the applicants hereby submit a certified translation for each of the priority documents (of the present application), German Patent Appl. No. DE 199 56 133.8, filed November 23, 1999 and German Patent Appl. No. DE 100 11 922.0, filed March 11, 2000. The applicants respectfully request entry of the enclosed documents into the official record of the above-identified patent application.

Respectfully submitted,

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Enclosure: Certified Translations of German Priority Documents

FEDERAL REPUBLIC OF GERMANY

Certificate of Priority for Filing of a Patent Application

Filing number:	100 11 922.0
Filing date:	11th March 2000
Applicant/Proprietor:	Degussa-Hüls Aktiengesellschaft, Frankfurt am Main/Germany
Title:	Novel nucleotide sequences coding for the pfkA gene
Priority:	23.11.1999 DE 199 56 133.8
IPC:	C 07 H, C 12 N, C 12 P

The attached papers are a true and accurate reproduction of the original documents for this patent application.

Munich, 26th September 2000
On behalf of the President of the German Patent and Trade Mark Office

(signature)

Dzierzon

Novel nucleotide sequences coding for the pfkA gene

The present invention provides nucleotide sequences coding for the pfkA gene and a process for the fermentative production of amino acids, in particular L-lysine, using coryneform bacteria in which the pfkA gene is amplified.

Prior art

Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, but in particular in animal nutrition.

It is known that amino acids are produced by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. Due to their great significance, efforts are constantly being made to improve the production process. Improvements to the process may relate to measures concerning fermentation technology, for example stirring and oxygen supply, or to the composition of the nutrient media, such as for example sugar concentration during fermentation, or to working up of the product by, for example, ion exchange chromatography, or to the intrinsic performance characteristics of the microorganism itself.

The performance characteristics of these microorganisms are improved using methods of mutagenesis, selection and mutant selection. In this manner, strains are obtained which are resistant to antimetabolites, such as for example the lysine analogue S-(2-aminoethyl)cysteine, or are auxotrophic for regulatorily significant metabolites and produce L-amino acids, such as for example L-lysine.

For some years, methods of recombinant DNA technology have likewise been used to improve strains of Corynebacterium which produce amino acids by amplifying individual biosynthesis genes and investigating the effect on amino

acid production. Review articles on this subject may be found inter alia in Kinoshita ("Glutamic Acid Bacteria", in: Biology of Industrial Microorganisms, Demain and Solomon (Eds.), Benjamin Cummings, London, UK, 1985, 115-142), Hilliger (BioTec 2, 40-44 (1991)), Eggeling (Amino Acids 6:261-272 (1994)), Jetten and Sinskey (Critical Reviews in Biotechnology 15, 73-103 (1995)) and Sahm et al. (Annuals of the New York Academy of Science 782, 25-39 (1996)).

Object of the invention

The inventors set themselves the object of providing novel measures for the improved fermentative production of amino acids, in particular L-lysine.

Description of the invention

Amino acids, in particular L-lysine, are used in human medicine, in the pharmaceuticals industry and in particular in animal nutrition. There is accordingly general interest in providing novel improved processes for the production of amino acids, in particular L-lysine.

Any subsequent mention of L-lysine or lysine should be taken to mean not only the base, but also salts, such as for example lysine monohydrochloride or lysine sulfate.

The invention provides an isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence selected from the group

a) polynucleotide which is at least 70% identical to a polynucleotide which codes for a polypeptide containing the amino acid sequence of SEQ ID no. 2,

b) polynucleotide which codes for a polypeptide which contains an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID no. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c).

The present invention also provides the polynucleotide according to claim 1, wherein it preferably comprises replicable DNA containing:

(i) the nucleotide sequence shown in SEQ ID no. 1, or

(ii) at least one sequence which matches the sequence (i) within the degeneration range of the genetic code, or

(iii) at least one sequence which hybridises with the complementary sequence to sequence (i) or (ii) and optionally

(iv) functionally neutral sense mutations in (i).

The present invention also provides

a polynucleotide according to claim 4, containing the nucleotide sequence as shown in SEQ ID no. 1,

a polynucleotide according to claim 6 which codes for a polypeptide which contains the amino acid sequence as shown in SEQ ID no. 2,

a vector containing the polynucleotide according to claim 1, in particular a shuttle vector or plasmid vector

and coryneform bacteria acting as host cell which contain the vector.

The present invention also provides polynucleotides which substantially consist of a polynucleotide sequence, which are obtainable by screening by means of hybridisation of a suitable gene library, which contains the complete gene having the polynucleotide sequence according to SEQ ID no. 1, with a probe which contains the sequence of the stated polynucleotide according to SEQ ID no. 1, or a fragment thereof, and isolation of the stated DNA sequence.

Polynucleotide sequences according to the invention are suitable as hybridisation probes for RNA, cDNA and DNA in order to isolate full length cDNA which code for phosphofructokinase and to isolate such cDNA or genes, the

sequence of which exhibits a high level of similarity with that of the phosphofructokinase gene.

Polynucleotide sequences according to the invention are furthermore suitable as primers for the production of DNA of genes which code for phosphofructokinase by the polymerase chain reaction (PCR).

Such oligonucleotides acting as probes or primers contain at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides having a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated from its natural environment.

"Polynucleotide" generally relates to polyribonucleotides and polydeoxyribonucleotides, wherein the RNA or DNA may be unmodified or modified.

"Polypeptides" are taken to mean peptides or proteins which contain two or more amino acids connected by peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID no. 2, in particular those having the biological activity of phosphofructokinase and also those, which are at least 70%, preferably at least 80%, identical to the polypeptide according to SEQ ID no. 2 and in particular are 90% to 95% identical to the polypeptide according to SEQ ID no. 2 and exhibit the stated activity.

The invention furthermore relates to a process for the fermentative production of amino acids, in particular L-lysine, using coryneform bacteria, which in particular already produce an amino acid and in which the nucleotide sequences which code for the pfkA gene are amplified, in particular overexpressed.

In this connection, the term "amplification" describes the increase in the intracellular activity of one or more enzymes in a microorganism, which enzymes are coded by the corresponding DNA, for example by increasing the copy number of the gene or genes, by using a strong promoter or a gene which codes for a corresponding enzyme having elevated activity and optionally by combining these measures.

The microorganisms, provided by the present invention, may produce L-amino acids, in particular L-lysine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms may comprise representatives of the coryneform bacteria in particular of the genus Corynebacterium. Within the genus Corynebacterium, the species Corynebacterium glutamicum may in particular be mentioned, which is known in specialist circles for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum, are for example the known wild type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-lysine producing mutants or strains produced therefrom, such as for example

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708

Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464 and
Corynebacterium glutamicum DSM5715.

The inventors succeeded in isolating the novel pfkA gene, which codes for the enzyme phosphofructokinase (EC 2.7.1.11), from C. glutamicum.

The pfkA gene or also other genes from C. glutamicum are isolated by initially constructing a gene library of this microorganism in E. coli. The construction of gene libraries is described in generally known textbooks and manuals. Examples which may be mentioned are the textbook by Winnacker, Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook et al., Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). One very well known gene library is that of E. coli K-12 strain W3110, which was constructed by Kohara et al. (Cell 50, 495-508 (1987)) in λ-vectors. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of C. glutamicum ATCC13032, which was constructed using the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326, 1992)) also describe a gene library of C. glutamicum ATCC 13032, using cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)). A gene library of C. glutamicum in E. coli may also be produced using plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are in particular those E. coli strains with restriction and recombination defects. One example of such a strain is the strain DH5αmcr, which has been described by Grant et al. (Proceedings of the

National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the assistance of cosmids may then in turn be sub-cloned in usual vectors suitable for sequencing and then be sequenced, as described, for example, in Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The novel DNA sequence from C. glutamicum which codes for the pfkA gene and, as SEQ ID no. 1, is provided by the present invention, was obtained in this manner. The amino acid sequence of the corresponding protein was furthermore deduced from the above DNA sequence using the methods described above. SEQ ID no. 2 shows the resultant amino acid sequence of the product of the pfkA gene.

Coding DNA sequences arising from the degeneracy of the genetic code are also provided by the present invention. DNA sequences which hybridise with SEQ ID no. 1 or parts of SEQ ID no. 1 are also provided by the invention. Conservative substitutions of amino acids in proteins, for example the substitution of glycine for alanine or of aspartic acid for glutamic acid, are known in specialist circles as "sense mutations", which result in no fundamental change in activity of the protein, i.e. they are functionally neutral. It is furthermore known that changes to the N and/or C terminus of a protein do not substantially impair or may even stabilise the function thereof. The person skilled in the art will find information in this connection inter alia in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences arising in a corresponding manner from SEQ ID no. 2 are also provided by the present invention.

Similarly, DNA sequences which hybridise with SEQ ID no. 1 or portions of SEQ ID no. 1 are also provided by the present invention. Finally, DNA sequences produced by the polymerase chain reaction (PCR) using primers obtained from SEQ ID no. 1 are also provided by the present invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

The person skilled in the art may find instructions for identifying DNA sequences by means of hybridisation inter alia in the manual "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The person skilled in the art may find instructions for amplifying DNA sequences using the polymerase chain reaction (PCR) inter alia in the manual by Gait, Oligonucleotide synthesis: a practical approach (IRL Press, Oxford, UK, 1984) and in Newton & Graham, PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

The inventors discovered that coryneform bacteria produce L-amino acids, in particular L-lysine, in an improved manner once the pfkA has been overexpressed.

Overexpression may be achieved by increasing the copy number of the corresponding genes or by mutating the promoter and regulation region or the ribosome-binding site located upstream from the structural gene. Expression cassettes incorporated upstream from the structural gene act in the same manner. It is additionally possible to increase expression during fermentative L-lysine production by means of inducible promoters. Expression is also improved by measures to extend the lifetime of the mRNA. Enzyme activity is moreover amplified by preventing degradation of the enzyme protein. The genes or gene constructs may either be present in plasmids in a variable copy number or be integrated in the chromosome and

amplified. Alternatively, overexpression of the genes concerned may also be achieved by modifying the composition of the nutrient media and culture conditions.

The person skilled in the art will find guidance in this connection inter alia in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European patent EPS 0 472 869, in US patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in patent application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in Japanese published patent application JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, the pfkA gene according to the invention was overexpressed with the assistance of plasmids.

Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as for example pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as for example those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891) may be used in the same manner.

Further suitable plasmid vectors are those with the assistance of which gene amplification may be performed by

integration into the chromosome, as has for example been described by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for the duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned into a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Vectors which may be considered are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The conjugation method is described, for example, in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Transformation methods are described, for example, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of "crossing over", the resultant strain contains at least two copies of the gene in question.

It may additionally be advantageous for the production of amino acids, in particular L-lysine, to amplify or overexpress not only the pfkA gene, but also one or more enzymes of the particular biosynthetic pathway, of glycolysis, of anaplerotic metabolism, of the citric acid cycle or of amino acid export.

For the production of L-lysine, for example, it is thus possible simultaneously to overexpress one or more genes selected from the group

- the dapA gene which codes for dihydropicolinate synthase (EP-B 0 197 335), or
- the gap gene which codes for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or
- the tpi gene which codes for triosephosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or
- the pyc gene which codes for pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), or
- the lysE gene which codes for lysine export (DE-A-195 48 222).

It may furthermore be advantageous for the production of amino acids, in particular L-lysine, in addition to amplifying the pfkA gene, simultaneously to attenuate

- the pck gene which codes for phosphoenolpyruvate carboxykinase (DE 199 50 409.1, DSM 13047) and/or
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969).

It may furthermore be advantageous for the production of amino acids, in particular L-lysine, in addition to overexpressing the pfkA gene, to suppress unwanted secondary reactions (Nakayama: "Breeding of Amino Acid

Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

For the purposes of amino acid production, in particular of L-lysine, the microorganisms produced according to the invention may be cultured continuously or discontinuously using the batch process or the fed batch process or repeated fed batch process. A summary of known culture methods is given in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must adequately satisfy the requirements of the particular strains. Culture media for various microorganisms are described in "Manual of Methods for General Bacteriology" from the American Society for Bacteriology (Washington D.C., USA, 1981). Carbon sources which may be used are sugars and carbohydrates, such as glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose for example, oils and fats, such as soya oil, sunflower oil, peanut oil and coconut oil for example, fatty acids, such as palmitic acid, stearic acid and linoleic acid for example, alcohols, such as glycerol and ethanol for example, and organic acids, such as acetic acid for example. These substances may be used individually or as a mixture. Nitrogen sources which may be used comprise organic compounds containing nitrogen, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya flour and urea or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or as a mixture. Phosphorus sources which may be used are phosphoric acid,

potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding salts containing sodium. The culture medium has additionally to contain salts of metals, such as magnesium sulfate or iron sulfate for example, which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins may also be used in addition to the above-stated substances. Suitable precursors may furthermore be added to the culture medium. The stated feed substances may be added to the culture as a single batch or be fed appropriately during culturing.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds, such as phosphoric acid or sulfuric acid, are used appropriately to control the pH of the culture. Foaming may be controlled by using antifoaming agents such as fatty acid polyglycol esters for example. Plasmid stability may be maintained by the addition to the medium of suitable selectively acting substances, for example antibiotics. Oxygen or oxygen-containing gas mixtures, such as air for example, are introduced into the culture in order to maintain aerobic conditions. The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C. The culture is continued until the maximum quantity of lysine has formed. This aim is normally achieved within 10 to 160 hours.

Analysis of L-lysine may be performed by anion exchange chromatography with subsequent ninhydrin derivatisation, as described in Spackman et al. (Analytical Chemistry, 30, (1958), 1190).

The purpose of the process according to the invention is the fermentative production of amino acids, in particular L-lysine.

The pfkA gene has been deposited in strain DSM 5715 under the designation DSM 5715/pT-pfkAexp with the registration number DSM 13253.

Examples

The present invention is illustrated in greater detail by the following practical examples.

Example 1

Production of a genomic cosmid gene library from Corynebacterium glutamicum ATCC13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described in Tauch et al., (1995, Plasmid 33:168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, code no. 1758250). The DNA of cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), purchased from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, code no. 27-0948-02) and also dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, code no. 27-0868-04). Cosmid DNA treated in this manner was mixed with the treated ATCC 13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4 DNA Ligase, code no. 27-0870-04). The ligation mixture was then packed in phages using Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, code no. 200217). E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) was infected by

suspending the cells in 10 mM $MgSO_4$ and mixing them with an aliquot of the phage suspension. The cosmid library was infected and titred as described in Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100µg/ml of ampicillin. After overnight incubation at 37°C, individual recombinant clones were selected.

Example 2

Isolation and sequencing of the pfkA gene

Cosmid DNA from an individual colony was isolated in accordance with the manufacturer's instructions using the Qiaprep Spin Miniprep Kit (product no. 27106, Qiagen, Hilden, Germany) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, product no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, product no. 1758250). Once separated by gel electrophoresis, the cosmid fragments of a size of 1500 to 2000 bp were isolated using the QiaExII Gel Extraction Kit (product no. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1 purchased from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, product no. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, product no. 27-0868-04). Ligation of the cosmid fragments into the sequencing vector pZero-1 was performed as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then

electroporated into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) and plated out onto LB agar (Lennox, 1955, Virology, 1:190) with 50 µg/ml of Zeocin. Plasmids of the recombinant clones were prepared using the Biorobot 9600 (product no. 900200, Qiagen, Hilden, Germany). Sequencing was performed using the dideoxy chain termination method according to Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) as modified by Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (product no. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the sequencing reaction was performed in a "Rotiphorese NF" acrylamide/bisacrylamide gel (29:1) (product no. A124.1, Roth, Karlsruhe, Germany) using the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The resultant raw sequence data were then processed using the Staden software package (1986, Nucleic Acids Research, 14:217-231), version 97-0. The individual sequences of the pZero 1 derivatives were assembled into a cohesive contig. Computer-aided coding range analysis was performed using XNIP software (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analysis was performed using the "BLAST search programs" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402), against the non-redundant database of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resultant nucleotide sequence is stated in SEQ ID no. 1. Analysis of the nucleotide sequence revealed an open reading frame of 1029 base pairs, which was designated the

pfkA gene. The pfkA gene codes for a protein of 343 amino acids.

SEQUENCE LISTING

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tgatgggttt aatatggaag ac atg cga att gct act ctc acg tca ggc ggc 172
Met Arg Ile Ala Thr Leu Thr Ser Gly Gly
1 5 10

gac tgc ccc gga cta aac gcc gtc atc cga gga atc gtc cgc aca gcc 220
Asp Cys Pro Gly Leu Asn Ala Val Ile Arg Gly Ile Val Arg Thr Ala
15 20 25

agc aat gaa ttt ggc tcc acc gtc gtt ggt tat caa gac ggt tgg gaa 268
Ser Asn Glu Phe Gly Ser Thr Val Val Gly Tyr Gln Asp Gly Trp Glu
30 35 40

gga ctg tta ggc gat cgt cgc gta cag ctg tat gac gat gaa gat att 316
Gly Leu Leu Gly Asp Arg Arg Val Gln Leu Tyr Asp Asp Glu Asp Ile
45 50 55

gac cga atc ctc ctt cga ggc ggc acc att ttg ggc act ggt cgc ctc 364
Asp Arg Ile Leu Leu Arg Gly Gly Thr Ile Leu Gly Thr Gly Arg Leu
60 65 70

cat ccg gac aag ttt aag gcc gga att gat cag att aag gcc aac tta 412
His Pro Asp Lys Phe Lys Ala Gly Ile Asp Gln Ile Lys Ala Asn Leu
75 80 85 90

gaa gac gcc ggc atc gat gcc ctt atc cca atc ggt ggc gaa gga acc 460
Glu Asp Ala Gly Ile Asp Ala Leu Ile Pro Ile Gly Gly Glu Gly Thr
95 100 105

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ctg aag ggt gcc aag tgg ctg tct gat aac ggt atc cct gtt gtc ggt   508
Leu Lys Gly Ala Lys Trp Leu Ser Asp Asn Gly Ile Pro Val Val Gly
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gtc cca aag acc att gac aat gac gtg aat ggc act gac ttc acc ttc   556
Val Pro Lys Thr Ile Asp Asn Asp Val Asn Gly Thr Asp Phe Thr Phe
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ggt ttc gat act gct gtg gca gtg gct acc gac gct gtt gac cgc ctg   604
Gly Phe Asp Thr Ala Val Ala Val Ala Thr Asp Ala Val Asp Arg Leu
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cac acc acc gct gaa tct cac aac cgt gtg atg atc gtg gag gtc atg   652
His Thr Thr Ala Glu Ser His Asn Arg Val Met Ile Val Glu Val Met
155                 160                 165                 170

ggc cgc cac gtg ggt tgg att gct ctg cac gca ggt atg gcc ggc ggt   700
Gly Arg His Val Gly Trp Ile Ala Leu His Ala Gly Met Ala Gly Gly
                175                 180                 185

gct cac tac acc gtt att cca gaa gta cct ttc gat att gca gag atc   748
Ala His Tyr Thr Val Ile Pro Glu Val Pro Phe Asp Ile Ala Glu Ile
            190                 195                 200

tgc aag gcg atg gaa cgt cgc ttc cag atg ggc gag aag tac ggc att   796
Cys Lys Ala Met Glu Arg Arg Phe Gln Met Gly Glu Lys Tyr Gly Ile
        205                 210                 215

atc gtc gtt gcg gaa ggt gcg ttg cca cgc gaa ggc acc atg gag ctt   844
Ile Val Val Ala Glu Gly Ala Leu Pro Arg Glu Gly Thr Met Glu Leu
    220                 225                 230

cgt gaa ggc cac att gac cag ttc ggt cac aag acc ttc acg gga att   892
Arg Glu Gly His Ile Asp Gln Phe Gly His Lys Thr Phe Thr Gly Ile
235                 240                 245                 250

gga cag cag atc gct gat gag atc cac gtg cgc ctc ggc cac gat gtt   940
Gly Gln Gln Ile Ala Asp Glu Ile His Val Arg Leu Gly His Asp Val
                255                 260                 265

cgt acg acc gtt ctt ggc cac att caa cgt ggt gga acc cca act gct   988
Arg Thr Thr Val Leu Gly His Ile Gln Arg Gly Gly Thr Pro Thr Ala
            270                 275                 280

ttc gac cgt gtt ctg gcc act cgt tat ggt gtt cgt gca gct cgt gcg   1036
Phe Asp Arg Val Leu Ala Thr Arg Tyr Gly Val Arg Ala Ala Arg Ala
        285                 290                 295

tgc cat gag gga agc ttt gac aag gtt gtt gct ttg aag ggt gag agc   1084
Cys His Glu Gly Ser Phe Asp Lys Val Val Ala Leu Lys Gly Glu Ser
    300                 305                 310

att gag atg atc acc ttt gaa gaa gca gtc gga acc ttg aag gaa gtt   1132
Ile Glu Met Ile Thr Phe Glu Glu Ala Val Gly Thr Leu Lys Glu Val
315                 320                 325                 330
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35 40 45

Arg Val Gln Leu Tyr Asp Asp Glu Asp Ile Asp Arg Ile Leu Leu Arg
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Gly Gly Thr Ile Leu Gly Thr Gly Arg Leu His Pro Asp Lys Phe Lys
65 70 75 80

Ala Gly Ile Asp Gln Ile Lys Ala Asn Leu Glu Asp Ala Gly Ile Asp
85 90 95

Ala Leu Ile Pro Ile Gly Gly Glu Gly Thr Leu Lys Gly Ala Lys Trp
100 105 110

Leu Ser Asp Asn Gly Ile Pro Val Val Gly Val Pro Lys Thr Ile Asp
115 120 125

Asn Asp Val Asn Gly Thr Asp Phe Thr Phe Gly Phe Asp Thr Ala Val
130 135 140

Ala Val Ala Thr Asp Ala Val Asp Arg Leu His Thr Thr Ala Glu Ser
145 150 155 160

His Asn Arg Val Met Ile Val Glu Val Met Gly Arg His Val Gly Trp
165 170 175

Ile Ala Leu His Ala Gly Met Ala Gly Gly Ala His Tyr Thr Val Ile
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Pro Glu Val Pro Phe Asp Ile Ala Glu Ile Cys Lys Ala Met Glu Arg
195 200 205

Arg Phe Gln Met Gly Glu Lys Tyr Gly Ile Ile Val Val Ala Glu Gly
210 215 220

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Ala Leu Pro Arg Glu Gly Thr Met Glu Leu Arg Glu Gly His Ile Asp
225                 230                 235                 240

Gln Phe Gly His Lys Thr Phe Thr Gly Ile Gly Gln Gln Ile Ala Asp
                245                 250                 255

Glu Ile His Val Arg Leu Gly His Asp Val Arg Thr Thr Val Leu Gly
            260                 265                 270

His Ile Gln Arg Gly Gly Thr Pro Thr Ala Phe Asp Arg Val Leu Ala
        275                 280                 285

Thr Arg Tyr Gly Val Arg Ala Ala Arg Ala Cys His Glu Gly Ser Phe
    290                 295                 300

Asp Lys Val Val Ala Leu Lys Gly Glu Ser Ile Glu Met Ile Thr Phe
305                 310                 315                 320

Glu Glu Ala Val Gly Thr Leu Lys Glu Val Pro Phe Glu Arg Trp Val
                325                 330                 335

Thr Ala Gln Ala Met Phe Gly
            340
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Patent Claims

1. Isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence selected from the group

   a) polynucleotide which is at least 70% identical to a polynucleotide which codes for a polypeptide containing the amino acid sequence of SEQ ID no. 2,

   b) polynucleotide which codes for a polypeptide which contains an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID no. 2,

   c) polynucleotide which is complementary to the polynucleotides of a) or b), and

   e) polynucleotide containing at least 15 successive bases of the polynucleotide sequence of a), b) or c).

2. A polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA replicable in coryneform bacteria.

3. A polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.

4. A polynucleotide as claimed in claim 2, containing the nucleic acid sequence as shown in SEQ ID no. 1.

5. Replicable DNA as claimed in claim 2, containing

   (i) the nucleotide sequence shown in SEQ ID no. 1, or

(ii) at least one sequence which matches the sequence (i) within the degeneration range of the genetic code, or

(iii) at least one sequence which hybridises with the complementary sequence to sequence (i) or (ii) and optionally

(iv) functionally neutral sense mutations in (i).

6. A polynucleotide sequence as claimed in claim 2 which codes for a polypeptide which contains the amino acid sequence shown in SEQ ID no. 2.

7. A process for the fermentative production of L-amino acids, in particular L-lysine,
wherein the following steps are performed:
a) fermentation of L-amino acid producing coryneform bacteria in which at least the pfkA gene or nucleotide sequences coding therefor is/are amplified, in particular overexpressed,
b) accumulation of the L-amino acid in the medium or in the cells of the bacteria and
c) isolation of the L-amino acid.

8. A process as claimed in claim 7,
wherein bacteria are used in which further genes of the biosynthetic pathway of the desired L-amino acid are additionally amplified.

9. A process as claimed in claim 7,
wherein bacteria are used in which the metabolic pathways which reduce the formation of L-lysine are at least partially suppressed.

10. A process as claimed in claim 7,
wherein a strain transformed with a plasmid vector is

used and the plasmid vector bears the nucleotide sequences which code for the pfkA gene.

11. A process as claimed in one or more of claims 7 to 10, wherein coryneform bacteria are used which produce L-lysine.

12. A process as claimed in claim 6, wherein bacteria are fermented for the production of lysine in which one or more of the genes selected from the group

12.1 the dapA gene which codes for dihydropicolinate synthase,

12.2 the pyc gene, which codes for pyruvate carboxylase,

12.3 the tpi gene, which codes for triosephosphate isomerase,

12.4 the dapE gene, which codes for succinyldiaminopimelate desuccinylase,

12.5 the gap gene, which codes for glyceraldehyde 3-phosphate dehydrogenase,

12.6 the pgk gene, which codes for 3-phosphoglycerate kinase,

12.7 the lysE gene, which codes for lysine export,

is/are simultaneously enhanced, in particular overexpressed or amplified.

13. A process as claimed in claim 9, wherein bacteria are fermented for the production of L-lysine in which one or more of the genes selected from the group

13.1 the pck gene, which codes for phosphoenolpyruvate carboxykinase,

13.2 the pgi gene, which codes for glucose 6-phosphate isomerase.

14. A process as claimed in one or more of the preceding claims,
wherein microorganisms of the genus Corynebacterium glutamicum are used.

15. Use of polynucleotide sequences as claimed in claim 1 as primers for the production of DNA of genes which code for phosphofructokinase by the polymerase chain reaction.

16. Use of polynucleotide sequences as claimed in claim 1 as hybridisation probes.

Novel nucleotide sequences coding for the pfkA gene

Abstract

The present invention provides an isolated polynucleotide containing a polynucleotide sequence selected from the group

a) polynucleotide which is at least 70% identical to a polynucleotide which codes for a polypeptide containing the amino acid sequence of SEQ ID no. 2,

b) polynucleotide which codes for a polypeptide which contains an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID no. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

Process for the fermentative production of L-amino acids with amplification of the pfkA gene and use as primer or hybridisation probe.